photolysis with, for example, a solution of di-isopropylethylamine (DIEA) in methylene chloride for about 5 minutes.

The first monomer is then placed at the first locations on the substrate. After irradiation, the slide is removed, treated in bulk, and then reinstalled in the flow cell. Alternatively, a fluid containing the first monomer, preferably also protected by a protective group, is circulated through the cavity by way of pump **116**. If, for example, it is desired to attach the amino acid Y to the substrate at the first locations, the amino acid Y (bearing a protective group on its α-nitrogen), along with reagents used to render the monomer reactive, and/or a carrier, is circulated from a storage container **118**, through the pump, through the cavity, and back to the inlet of the pump.

The monomer carrier solution is, in a preferred embodiment, formed by mixing of a first solution (referred to herein as solution "A") and a second solution (referred to herein as solution "B"). Table 2 provides an illustration of a mixture which may be used for solution A.

TABLE 2

Representative Monomer Carrier Solution "A"	
100 mg	NVOC amino protected amino acid
37 mg	HOBT (1-Hydroxybenzotriazole)
250 μl	DMF (Dimethylformamide)
86 μl	DIEA (Diisopropylethylamine)

The composition of solution B is illustrated in Table 3. Solutions A and B are mixed and allowed to react at room temperature for about 8 minutes, then diluted with 2 ml of DMF, and 500 μl are applied to the surface of the slide or the solution is circulated through the reactor system and allowed to react for about 2 hours at room temperature. The slide is then washed with DMF, methylene chloride and ethanol.

TABLE 3

Representative Monomer Carrier Solution "B"	
250 μl	DMF
111 mg	BOP (Benzotriazolyl-n-oxy-tris (dimethylamino) phosphoniumhexafluorophosphate)

As the solution containing the monomer to be attached is circulated through the cavity, the amino acid or other monomer will react at its carboxy terminus with amino groups on the regions of the substrate which have been deprotected. Of course, while the invention is illustrated by way of circulation of the monomer through the cavity, the invention could be practiced by way of removing the slide from the reactor and submersing it in an appropriate monomer solution.

After addition of the first monomer, the solution containing the first amino acid is then purged from the system. After circulation of a sufficient amount of the DMF/methylene chloride such that removal of the amino acid can be assured (e.g., about 50× times the volume of the cavity and carrier lines), the mask or substrate is repositioned, or a new mask is utilized such that second regions on the substrate will be exposed to light and the light **124** is engaged for a second exposure. This will deprotect second regions on the substrate and the process is repeated until the desired polymer sequences have been synthesized.

The entire derivatized substrate is then exposed to a receptor of interest, preferably labeled with, for example, a fluorescent marker, by circulation of a solution or suspension of the receptor through the cavity or by contacting the surface of the slide in bulk. The receptor will preferentially bind to certain regions of the substrate which contain complementary sequences.

Antibodies are typically suspended in what is commonly referred to as "supercocktail," which may be, for example, a solution of about 1% BSA (bovine serum albumin), 0.5% Tween in PBS (phosphate buffered saline) buffer. The antibodies are diluted into the supercocktail buffer to a final concentration of, for example, about 0.1 to 4 μg/ml.

FIG. **8B** illustrates an alternative preferred embodiment of the reactor shown in FIG. **8A**. According to this embodiment, the mask **128** is placed directly in contact with the substrate. Preferably, the etched portion of the mask is placed face down so as to reduce the effects of light dispersion. According to this embodiment, the imaging lenses **120** and **126** are not necessary because the mask is brought into close proximity with the substrate.

For purposes of increasing the signal-to-noise ratio of the technique, some embodiments of the invention provide for exposure of the substrate to a first labeled or unlabeled receptor followed by exposure of a labeled, second receptor (e.g., an antibody) which binds at multiple sites on the first receptor. If, for example, the first receptor is an antibody derived from a first species of an animal, the second receptor is an antibody derived from a second species directed to epitopes associated with the first species. In the case of a mouse antibody, for example, fluorescently labeled goat antibody or antiserum which is antimouse may be used to bind at multiple sites on the mouse antibody, providing several times the fluorescence compared to the attachment of a single mouse antibody at each binding site. This process may be repeated again with additional antibodies (e.g., goat-mouse-goat, etc.) for further signal amplification.

In preferred embodiments an ordered sequence of masks is utilized. In some embodiments it is possible to use as few as a single mask to synthesize all of the possible polymers of a given monomer set.

If, for example, it is desired to synthesize all 16 dinucleotides from four bases, a 1 cm square synthesis region is divided conceptually into 16 boxes, each 0.25 cm wide. Denote the four monomer units by A, B, C, and D. The first reactions are carried out in four vertical columns, each 0.25 cm wide. The first mask exposes the left-most column of boxes, where A is coupled. The second mask exposes the next column, where B is coupled; followed by a third mask, for the C column; and a final mask that exposes the right-most column, for D. The first, second, third, and fourth masks may be a single mask translated to different locations.

The process is repeated in the horizontal direction for the second unit of the dimer. This time, the masks allow exposure of horizontal rows, again 0.25 cm wide. A, B, C, and D are sequentially coupled using masks that expose horizontal fourths of the reaction area. The resulting substrate contains all 16 dinucleotides of four bases.

The eight masks used to synthesize the dinucleotide are related to one another by translation or rotation. In fact, one mask can be used in all eight steps if it is suitably rotated and translated. For example, in the example above, a mask with a single transparent region could be sequentially used to expose each of the vertical columns, translated 90°, and then sequentially used to allow exposure of the horizontal rows.

Tables 4 and 5 provide a simple computer program in Quick Basic for planning a masking program and a sample output, respectively, for the synthesis of a polymer chain of three monomers ("residues") having three different monomers in the first level, four different monomers in the second level, and five different monomers in the third level in a

striped pattern. The output of the program is the number of cells, the number of "stripes" (light regions) on each mask, and the amount of translation required for each exposure of the mask.

TABLE 4

Mask Strategy Program

```
DEFINT A-Z
DIM b(20), w(20), l(500)
F$ = "LPT1:"
OPEN f$ FOR OUTPUT AS #1
jmax = 3 'Number of residues
b(1) = 3: b(2) = 4: b(3) = 5 'Number of building blocks for res 1,2,3
g = 1: lmax(1) = 1
FOR j = 1 TO jmax: g= g * b(j): NEXT j
w(0) = 0: w(1) = g / b(l)
PRINT #1, "MASK2.BAS ", DATE$, TIME$: PRINT #1,
PRINT #1, USING "Number of residues=##"; jmax
FOR j = 1 TO jmax
PRINT #1, USING "      Residue ##      ##building blocks"; j; b(j)
NEXT j
PRINT #1, "
PRINT #1, USING "Number of cells=####"; g: PRINT #1,
FOR j = 2 TO jmax
lmax(j) = lmax(j - 1) * b(j - 1)
w(j) = w(j - 1) / b(j)
NEXT j
FOR j = 1 TO jmax
PRINT #1, USING "Mask for residue ##"; j: PRINT #1,
PRINT #1, USING "    Number of stripes =###"; lmax(j)
PRINT #1, USING "    Width of each stripe =###"; w(j)
FOR 1 = 1 TO lmax(j)
a = 1 + (1 - 1) * w(j - 1)
ae = a + w(j) - 1
PRINT #1, USING " Stripe ## begins at
location ### and ends at ###"; 1; a; ae
NEXT 1
PRINT #1,
PRINT #1, USING " For each of ## building blocks, translate mask by ##
cell(s)"; b(j); w(j),
PRINT #1, : PRINT #1, : PRINT #1,
NEXT j
```

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TABLE 5

Masking Strategy Output

```
Number of residues= 3
Residue 1                          3 building blocks
Residue 2                          4 building blocks
Residue 3                          5 building blocks
Number of cells= 60
Mask for residue 1

Number of stripes= 1
Width of each stripe= 20
Stripe 1 begins at location    1 and ends at 20
For each of 3 building blocks, translate mask by 20 cell(s)
Mask for residue 2

Number of stripes= 3
Width of each stripe= 5
Stripe 1 begins at location          1 and ends at 5
Stripe 2 begins at location         21 and ends at 25
Stripe 3 begins at location         41 and ends at 45
For each of 4 building blocks, translate mask by 5 cell(s)
Mask for residue 3

Number of stripes= 12
Width of each stripe= 1
Stripe  1 begins at location          1 and ends at  1
Stripe  2 begins at location          6 and ends at  6
Stripe  3 begins at location         11 and ends at 11
Stripe  4 begins at location         16 and ends at 16
Stripe  5 begins at location         21 and ends at 21
```

TABLE 5-continued

Masking Strategy Output

```
Stripe  6 begins at location         26 and ends at 26
Stripe  7 begins at location         31 and ends at 31
Stripe  8 begins at location         36 and ends at 36
Stripe  9 begins at location         41 and ends at 41
Stripe 10 begins at location         46 and ends at 46
Stripe 11 begins at location         51 and ends at 51
Stripe 12 begins at location         56 and ends at 56
For each of 5 building blocks, translate mask by 1 cell(s)
```

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V. Details of One Embodiment of A Fluorescent Detection Device

FIG. **9** illustrates a fluorescent detection device for detecting fluorescently labeled receptors on a substrate. A substrate **112** is placed on an x/y translation table **202**. In a preferred embodiment the x/y translation table is a model no. PM500-A1 manufactured by Newport Corporation. The x/y translation table is connected to and controlled by an appropriately programmed digital computer **204** which may be, for example, an appropriately programmed IBM PC/AT or AT compatible computer. Of course, other computer systems, special purpose hardware, or the like could readily be substituted for the AT computer used herein for illustration. Computer software for the translation and data collection functions described herein can be provided based on commercially available software including, for example, "Lab Windows" licensed by National Instruments, which is incorporated herein by reference for all purposes.

The substrate and x/y translation table are placed under a microscope **206** which includes one or more objectives **208**. Light (about 488 nm) from a laser **210**, which in some embodiments is a model no. 2020-05 argon ion laser manufactured by Spectraphysics, is directed at the substrate by a dichroic mirror **207** which passes greater than about 520 nm light but reflects 488 nm light. Dichroic mirror **207** may be, for example, a model no. FT510 manufactured by Carl Zeiss. Light reflected from the mirror then enters the microscope **206** which may be, for example, a model no. Axioscop 20 manufactured by Carl Zeiss. Fluorescein-marked materials on the substrate will fluoresce >488 nm light, and the fluoresced light will be collected by the microscope and passed through the mirror. The fluorescent light from the substrate is then directed through a wavelength filter **209** and, thereafter through an aperture plate **211**. Wavelength filter **209** may be, for example, a model no. OG530 manufactured by Melles Griot and aperture plate **211** may be, for example, a model no. 477352/477380 manufactured by Carl Zeiss.

The fluoresced light then enters a photomultiplier tube **212** which in some embodiments is a model no. R943-02 manufactured by Hamamatsu, the signal is amplified in preamplifier 214 and photons are counted by photon counter **216**. The number of photons is recorded as a function of the location in the computer **204**. Pre-Amp **214** may be, for example, a model no. SR440 manufactured by Stanford Research Systems and photon counter **216** may be a model no. SR400 manufactured by Stanford Research Systems. The substrate is then moved to a subsequent location and the process is repeated. In preferred embodiments the data are acquired every 1 to 100 μm with a data collection diameter of about 0.8 to 10 μm preferred. In embodiments with sufficiently high fluorescence, a CCD detector with broad-field illumination is utilized.

By counting the number of photons generated in a given area in response to the laser, it is possible to determine where

fluorescent marked molecules are located on the substrate. Consequently, for a slide which has a matrix of polypeptides, for example, synthesized on the surface thereof, it is possible to determine which of the polypeptides is complementary to a fluorescently marked receptor.

According to preferred embodiments, the intensity and duration of the light applied to the substrate is controlled by varying the laser power and scan stage rate for improved signal-to-noise ratio by maximizing fluorescence emission and minimizing background noise.

While the detection apparatus has been illustrated primarily herein with regard to the detection of marked receptors, the invention will find application in other areas. For example, the detection apparatus disclosed herein could be used in the fields of catalysis, DNA or protein gel scanning, and the like.

VI. Determination of Relative Binding Strength of Receptors

The signal-to-noise ratio of the present invention is sufficiently high that not only can the presence or absence of a receptor on a ligand be detected, but also the relative binding affinity of receptors to a variety of sequences can be determined.

In practice it is found that a receptor will bind to several peptide sequences in an array, but will bind much more strongly to some sequences than others. Strong binding affinity will be evidenced herein by a strong fluorescent or radiographic signal since many receptor molecules will bind in a region of a strongly bound ligand. Conversely, a weak binding affinity will be evidenced by a weak fluorescent or radiographic signal due to the relatively small number of receptor molecules which bind in a particular region of a substrate having a ligand with a weak binding affinity for the receptor. Consequently, it becomes possible to determine relative binding avidity (or affinity in the case of univalent interactions) of a ligand herein by way of the intensity of a fluorescent or radiographic signal in a region containing that ligand.

Semiquantitative data on affinities might also be obtained by varying washing conditions and concentrations of the receptor. This would be done by comparison to known ligand receptor pairs, for example.

VII. Examples

The following examples are provided to illustrate the efficacy of the inventions herein. All operations were conducted at about ambient temperatures and pressures unless indicated to the contrary.

A. Slide Preparation

Before attachment of reactive groups it is preferred to clean the substrate which is, in a preferred embodiment a glass substrate such as a microscope slide or cover slip. According to one embodiment the slide is soaked in an alkaline bath consisting of, for example, 1 liter of 95% ethanol with 120 ml of water and 120 grams of sodium hydroxide for 12 hours. The slides are then washed under running water and allowed to air dry, and rinsed once with a solution of 95% ethanol.

The slides are then aminated with, for example, aminopropyltriethoxysilane for the purpose of attaching amino groups to the glass surface on linker molecules, although any omega functionalized silane could also be used for this purpose. In one embodiment 0.1% aminopropyltriethoxysilane is utilized, although solutions with concentrations from 10^{-7}% to 10% may be used, with about 10^{-3}% to 2% preferred. A 0.1% mixture is prepared by adding to 100 ml of a 95% ethanol/5% water mixture, 100 microliters (μl) of aminopropyltriethoxysilane. The mixture is agitated at about ambient temperature on a rotary shaker for about 5 minutes. 500 μl of this mixture is then applied to the surface of one side of each cleaned slide. After 4 minutes, the slides are decanted of this solution and rinsed three times by dipping in, for example, 100% ethanol.

After the plates dry, they are placed in a 110–120° C. vacuum oven for about 20 minutes, and then allowed to cure at room temperature for about 12 hours in an argon environment. The slides are then dipped into DMF (dimethylformamide) solution, followed by a thorough washing with methylene chloride.

The aminated surface of the slide is then exposed to about 500 μl of, for example, a 30 millimolar (mM) solution of NVOC-GABA (gamma amino butyric acid) NHS (N-hydroxysuccinimide) in DMF for attachment of a NVOC-GABA to each of the amino groups.

The surface is washed with, for example, DMF, methylene chloride, and ethanol.

Any unreacted aminopropyl silane on the surface—that is, those amino groups which have not had the NVOC-GABA attached—are now capped with acetyl groups (to prevent further reaction) by exposure to a 1:3 mixture of acetic anhydride in pyridine for 1 hour. Other materials which may perform this residual capping function include trifluoroacetic anhydride, formicacetic anhydride, or other reactive acylating agents. Finally, the slides are washed again with DMF, methylene chloride, and ethanol.

B. Synthesis of Eight Trimers of "A" and "B"

FIG. **10** illustrates a possible synthesis of the eight trimers of the two-monomer set: gly, phe (represented by "A" and "B," respectively). A glass slide bearing silane groups terminating in 6-nitro-veratryloxycarboxamide (NVOC-NH) residues is prepared as a substrate. Active esters (pentafluorophenyl, OBt, etc.) of gly and phe protected at the amino group with NVOC are prepared as reagents. While not pertinent to this example, if side chain protecting groups are required for the monomer set, these must not be photoreactive at the wavelength of light used to protect the primary chain.

For a monomer set of size n, n×ρ cycles are required to synthesize all possible sequences of length ρ. A cycle consists of:

1. Irradiation through an appropriate mask to expose the amino groups at the sites where the next residue is to be added, with appropriate washes to remove the by-products of the deprotection.
2. Addition of a single activated and protected (with the same photochemically-removable group) monomer, which will react only at the sites addressed in step 1, with appropriate washes to remove the excess reagent from the surface.

The above cycle is repeated for each member of the monomer set until each location on the surface has been extended by one residue in one embodiment. In other embodiments, several residues are sequentially added at one location before moving on to the next location. Cycle times will generally be limited by the coupling reaction rate, now as short as 20 min in automated peptide synthesizers. This step is optionally followed by addition of a protecting group to stabilize the array for later testing. For some types of polymers (e.g., peptides), a final deprotection of the entire surface (removal of photoprotective side chain groups) may be required.

More particularly, as shown in FIG. **10**A, the glass **20** is provided with regions **22**, **24**, **26**, **28**, **30**, **32**, **34**, and **36**. Regions **30**, **32**, **34**, and **36** are masked, as shown in FIG. **10**B and the glass is irradiated and exposed to a reagent

containg "A" (e.g., gly), with the resulting structure shown in FIG. 10C. Thereafter, regions 22, 24, 26, and 28 are masked, the glass is irradiated (as shown in FIG. 10D) and exposed to a reagent containing "B" (e.g., phe), with the resulting structure shown in FIG. 10E. The process proceeds, consecutively masking and exposing the sections as shown until the structure shown in FIG. 10M is obtained. The glass is irradiated and the terminal groups are, optionally, capped by acetylation. As shown, all possible trimers of gly/phe are obtained.

In this example, no side chain protective group removal is necessary. If it is desired, side chain deprotection may be accomplished by treatment with ethanedithiol and trifluoroacetic acid.

In general, the number of steps needed to obtain a particular polymer chain is defined by:

$$n \times e \quad (1)$$

where:

n=the number of monomers in the basis set of monomers, and

e=the number of monomer units in a polymer chain.

Conversely, the synthesized number of sequences of length e will be:

$$n^e, \quad (2)$$

Of course, greater diversity is obtained by using masking strategies which will also include the synthesis of polymers having a length of less than e. If, in the extreme case, all polymers having a length less than or equal to e are synthesized, the number of polymers synthesized will be:

$$n^e + n^{e-1} + \ldots + n^1. \quad (3)$$

The maximum number of lithographic steps needed will generally be n for each "layer" of monomers, i.e., the total number of masks (and, therefore, the number of lithographic steps) needed will be n×e. The size of the transparent mask regions will vary in accordance with the area of the substrate available for synthesis and the number of sequences to be formed. In general, the size of the synthesis areas will be:

$$\text{size of synthesis areas} = (A)/(S)$$

where:

A is the total area available for synthesis; and

S is the number of sequences desired in the area.

It will be appreciated by those of skill in the art that the above method could readily be used to simultaneously produce thousands or millions of oligomers on a substrate using the photolithographic techniques disclosed herein. Consequently, the method results in the ability to practically test large numbers of, for example, di, tri, tetra, penta, hexa, hepta, octapeptides, dodecapeptides, or larger polypeptides (or correspondingly, polynucleotides).

The above example has illustrated the method by way of a manual example. It will of course be appreciated that automated or semi-automated methods could be used. The substrate would be mounted in a flow cell for automated addition and removal of reagents, to minimize the volume of reagents needed, and to more carefully control reaction conditions. Successive masks could be applied manually or automatically.

C. Synthesis of a Dimer of an Aminopropyl Group and a Fluorescent Group

In synthesizing the dimer of an aminopropyl group and a fluorescent group, a functionalized durapore membrane was used as a substrate. The durapore membrane was a polyvinylidine difluoride with aminopropyl groups. The aminopropyl groups were protected with the DDZ group by reaction of the carbonyl chloride with the amino groups, a reaction readily known to those of skill in the art. The surface bearing these groups was placed in a solution of THF and contacted with a mask bearing a checkerboard pattern of 1 mm opaque and transparent regions. The mask was exposed to ultraviolet light having a wavelength down to at least about 280 nm for about 5 minutes at ambient temperature, although a wide range of exposure times and temperatures may be appropriate in various embodiments of the invention. For example, in one embodiment, an exposure time of between about 1 and 5000 seconds may be used at process temperatures of between −70 and +50° C.

In one preferred embodiment, exposure times of between about 1 and 500 seconds at about ambient pressure are used. In some preferred embodiments, pressure above ambient is used to prevent evaporation.

The surface of the membrane was then washed for about 1 hour with a fluorescent label which included an active ester bound to a chelate of a lanthanide. Wash times will vary over a wide range of values from about a few minutes to a few hours. These materials fluoresce in the red and the green visible region. After the reaction with the active ester in the fluorophore was complete, the locations in which the fluorophore was bound could be visualized by exposing them to ultraviolet light and observing the red and the green fluorescence. It was observed that the derivatized regions of the substrate closely corresponded to the original pattern of the mask.

D. Demonstration of Signal Capability

Signal detection capability was demonstrated using a low-level standard fluorescent bead kit manufactured by Flow Cytometry Standarda and having model no. 824. This kit includes 5.8 μm diameter beads, each impregnated with a known number of fluorescein molecules.

One of the beads was placed in the illumination field on the scan stage as shown in FIG. 9 in a field of a laser spot which was initially shuttered. After being positioned in the illumination field, the photon detection equipment was turned on. The laser beam was unblocked and it interacted with the particle bead, which then fluoresced. Fluorescence curves of beads impregnated with 7,000; 13,000; and 29,000 fluorescein molecules, are shown in FIGS. 11A, 11B, and 11C respectively. On each curve, traces for beads without fluorescein molecules are also shown. These experiments were performed with 488 nm excitation, with 100 μW of laser power. The light was focused through a 40 power 0.75 NA objective.

The fluorescence intensity in all cases started off at a high value and then decreased exponentially. The fall-off in intensity is due to photobleaching of the fluorescein molecules. The traces of beads without fluorescein molecules are used for background subtraction. The difference in the initial exponential decay between labeled and nonlabeled beads is integrated to give the total number of photon counts, and this number is related to the number of molecules per bead. Therefore, it is possible to deduce the number of photons per fluorescein molecule that can be detected. For the curves illustrated in FIG. 11, this calculation indicates the radiation of about 40 to 50 photons per fluorescein molecule are detected.

E. Determination of the Number of Molecules Per Unit Area

Aminopropylated glass microscope slides prepared according to the methods discussed above were utilized in

order to establish the density of labeling of the slides. The free amino termini of the slides were reacted with FITC (fluorescein isothiocyanate) which forms a covalent linkage with the amino group. The slide is then scanned to count the number of fluorescent photons generated in a region which, using the estimated 40–50 photons per fluorescent molecule, enables the calculation of the number of molecules which are on the surface per unit area.

A slide with aminopropyl silane on its surface was immersed in a 1 mM solution of FITC in DMF for 1 hour at about ambient temperature. After reaction, the slide was washed twice with DMF and then washed with ethanol, water, and then ethanol again. It was then dried and stored in the dark until it was ready to be examined.

Through the use of curves similar to those shown in FIG. **11**, and by integrating the fluorescent counts under the exponentially decaying signal, the number of free amino groups on the surface after derivitization was determined. It was determined that slides with labeling densities of 1 fluoroscein per $10^3 \times 10^3$ to ~2×2 nm could be reproducibly made as the concentration of aminopropyltriethoxysilane varied from 10^{-5}% to 10^{-1}%.

F. Removal of NVOC and Attachment of A Fluorescent Marker

NVOC-GABA groups were attached as described above. The entire surface of one slide was exposed to light so as to expose a free amino group at the end of the gamma amino butyric acid. This slide, and a duplicate which was not exposed, were then exposed to fluorescein isothiocyanate (FITC).

FIG. **12**A illustrates the slide which was not exposed to light, but which was exposed to FITC. The units of the x axis are time and the units of the y axis are counts. The trace contains a certain amount of background fluorescence. The duplicate slide was exposed to 350 nm broadband illumination for about 1 minute (12 mW/cm^2, ~350 nm illumination), washed and reacted with FITC. The fluorescence curves for this slide are shown in FIG. **12**B. A large increase in the level of fluorescence is observed, which indicates photolysis has exposed a number of amino groups on the surface of the slides for attachment of a fluorescent marker.

G. Use of a Mask in Removal of NVOC

The next experiment was performed with a 0.1% aminopropylated slide. Light from a Hg-Xe arc lamp was imaged onto the substrate through a laser-ablated chrome-on-glass mask in direct contact with the substrate.

This slide was illuminated for approximately 5 minutes, with 12 mW of 350 nm broadband light and then reacted with the 1 mM FITC solution. It was put on the laser detection scanning stage and a graph was plotted as a two-dimensional representation of position color-coded for fluorescence intensity. The fluorescence intensity (in counts) as a function of location is given on the color scale to the right of FIG. **13**A for a mask having 100×100 μm squares.

The experiment was repeated a number of times through various masks. The fluorescence pattern for a 50 μm mask is illustrated in FIG. **13**B, for a 20 μm mask in FIG. **13**C, and for a 10 μm mask in FIG. **13**D. The mask pattern is distinct down to at least about 10 μm squares using this lithographic technique.

H. Attachment of YGGFL and Subsequent Exposure to Herz Antibody and Goat Antimouse

In order to establish that receptors to a particular polypeptide sequence would bind to a surface-bound peptide and be detected, Leu enkephalin was coupled to the surface and recognized by an antibody. A slide was derivatized with 0.1% amino propyl-triethoxysilane and protected with NVOC. A 500 μm checkerboard mask was used to expose the slide in a flow cell using backside contact printing. The Leu enkephalin sequence (H_2N-tyrosine, glycine, glycine, phenylalanine, leucine-CO_2H, otherwise referred to herein as YGGFL) was attached via its carboxy end to the exposed amino groups on the surface of the slide. The peptide was added in DMF solution with the BOP/HOBT/DIEA coupling reagents and recirculated through the flow cell for 2 hours at room temperature.

A first antibody, known as the Herz antibody, was applied to the surface of the slide for 45 minutes at 2 μg/ml in a supercocktail (containing 1% BSA and 1% ovalbumin also in this case). A second antibody, goat anti-mouse fluorescein conjugate, was then added at 2 μg/ml in the supercocktail buffer, and allowed to incubate for 2 hours.

The results of this experiment are provided in FIG. **14**. Again, this figure illustrates fluorescence intensity as a function of position. The fluorescence scale is shown on the right, according to the color coding. This image was taken at 10 μm steps. This figure indicates that not only can deprotection be carried out in a well defined pattern, but also that (1) the method provides for successful coupling of peptides to the surface of the substrate, (2) the surface of a bound peptide is available for binding with an antibody, and (3) that the detection apparatus capabilities are sufficient to detect binding of a receptor.

I. Monomer-by-Monomer Formation of YGGFL and Subsequent Exposure to Labeled Antibody

Monomer-by-monomer synthesis of YGGFL and GGFL in alternate squares was performed on a slide in a checkerboard pattern and the resulting slide was exposed to the Herz antibody. This experiment and the results thereof are illustrated in FIGS. **15**A, **15**B, **15**C, and **15**D.

In FIG. **15**A, a slide is shown which is derivatized with the aminopropyl group, protected in this case with t-BOC (t-butoxycarbonyl). The slide was treated with TFA to remove the t-BOC protecting group. E-aminocaproic acid, which was t-BOC protected at its amino group, was then coupled onto the aminopropyl groups. The aminocaproic acid serves as a spacer between the aminopropyl group and the peptide to be synthesized. The amino end of the spacer was deprotected and coupled to NVOC-leucine. The entire slide was then illuminated with 12 mW of 325 nm broadband illumination. The slide was then coupled with NVOC-phenylalanine and washed. The entire slide was again illuminated, then coupled to NVOC-glycine and washed. The slide was again illuminated and coupled to NVOC-glycine to form the sequence shown in the last portion of FIG. **15**A.

As shown in FIG. **15**B, alternating regions of the slide were then illuminated using a projection print using a 500×500 μm checkerboard mask; thus, the amino group of glycine was exposed only in the lighted areas. When the next coupling chemistry step was carried out, NVOC-tyrosine was added, and it coupled only at those spots which had received illumination. The entire slide was then illuminated to remove all the NVOC groups, leaving a checkerboard of YGGFL in the lighted areas and in the other areas, GGFL. The Herz antibody (which recognizes the YGGFL, but not GGFL) was then added, followed by goat anti-mouse fluorescein conjugate.

The resulting fluorescence scan is shown in FIG. **15**C, and the color coding for the fluorescence intensity is again given on the right. Dark areas contain the tetrapeptide GGFL, which is not recognized by the Herz antibody (and thus there is no binding of the goat anti-mouse antibody with fluores-

cein conjugate), and in the red areas YGGFL is present. The YGGFL pentapeptide is recognized by the Herz antibody and, therefore, there is antibody in the lighted regions for the fluorescein-conjugated goat anti-mouse to recognize.

Similar patterns are shown for a 50 μm mask used in direct contact ("proximity print") with the substrate in FIG. **15D**. Note that the pattern is more distinct and the corners of the checkerboard pattern are touching when the mask is placed in direct contact with the substrate (which reflects the increase in resolution using this technique).

J. Monomer-by-Monomer Synthesis of YGGFL and PGGFL

A synthesis using a 50 μm checkerboard mask similar to that shown in FIG. **15** was conducted. However, P was added to the GGFL sites on the substrate through an additional coupling step. P was added by exposing protected GGFL to light through a mask, and subsequence exposure to P in the manner set forth above. Therefore, half of the regions on the substrate contained YGGFL and the remaining half contained PGGFL.

The fluorescence plot for this experiment is provided in FIG. **16**. As shown, the regions are again readily discernable. This experiment demonstrates that antibodies are able to recognize a specific sequence and that the recognition is not length-dependent.

K. Monomer-by-Monomer Synthesis of YGGFL and YPGGFL

In order to further demonstrate the operability of the invention, a 50 μm checkerboard pattern of alternating YGGFL and YPGGFL was synthesized on a substrate using techniques like those set forth above. The resulting fluorescence plot is provided in FIG. **17**. Again, it is seen that the antibody is clearly able to recognize the YGGFL sequence and does not bind significantly at the YPGGFL regions.

L. Synthesis of an Array of Sixteen Different Amino Acid Sequences and Estimation of Relative Binding Affinity to Herz Antibody

Using techniques similar to those set forth above, an array of 16 different amino acid sequences (replicated four times) was synthesized on each of two glass substrates. The sequences were synthesized by attaching the sequence NVOC-GFL across the entire surface of the slides. Using a series of masks, two layers of amino acids were then selectively applied to the substrate. Each region had dimensions of 0.25 cm×0.0625 cm. The first slide contained amino acid sequences containing only L amino acids while the second slide contained selected D amino acids. FIGS. **18A** and **18B** illustrate a map of the various regions on the first and second slides, respectively. The patterns shown in FIGS. **18A** and **18B** were duplicated four times on each slide. The slides were then exposed to the Herz antibody and fluorescein-labeled goat anti-mouse.

FIG. **19** is a fluorescence plot of the first slide, which contained only L amino acids. Red indicates strong binding (149,000 counts or more) while black indicates little or no binding of the Herz antibody (20,000 counts or less). The bottom right-hand portion of the slide appears "cut off" because the slide was broken during processing. The sequence YGGFL is clearly most strongly recognized. The sequences YAGFL and YSGFL also exhibit strong recognition of the antibody. By contrast, most of the remaining sequences show little or no binding. The four duplicate portions of the slide are extremely consistent in the amount of binding shown therein.

FIG. **20** is a fluorescence plot of the second slide. Again, strongest binding is exhibited by the YGGFL sequence. Significant binding is also detected to YaGFL, YsGFL, and YpGFL. The remaining sequences show less binding with the antibody. Note the low binding efficiency of the sequence yGGFL.

Table 6 lists the various sequences tested in order of relative fluorescence, which provides information regarding relative binding affinity.

TABLE 6

Apparent Binding to Herz Ab	
L-a.a. Set	D-a.a. Set
YGGFL	YGGFL
YAGFL	YaGFL
YSGFL	YsGFL
LGGFL	YpGFL
FGGFL	fGGFL
YPGFL	yGGFL
LAGFL	faGFL
FAGFL	wGGFL
WGGFL	yaGFL
	fpGFL
	waGFL

VIII. Illustrative Alternative Embodiment

According to an alternative embodiment of the invention, the methods provide for attaching to the surface a caged binding member which in its caged form has a relatively low affinity for other potentially binding species, such as receptors and specific binding substances. Such techniques are more fully described in copending application Ser. No. 404,920, filed Sep. 8, 1989, and incorporated herein by reference for all purposes.

According to this alternative embodiment, the invention provides methods for forming predefined regions on a surface of a solid support, wherein the predefined regions are capable of immobilizing receptors. The methods make use of caged binding members attached to the surface to enable selective activation of the predefined regions. The caged binding members are liberated to act as binding members ultimately capable of binding receptors upon selective activation of the predefined regions. The activated binding members are then used to immobilize specific molecules such as receptors on the predefined region of the surface. The above procedure is repeated at the same or different sites on the surface so as to provide a surface prepared with a plurality of regions on the surface containing, for example, the same or different receptors. When receptors immobilized in this way have a differential affinity for one or more ligands, screenings and assays for the ligands can be conducted in the regions of the surface containing the receptors.

The alternative embodiment may make use of novel caged binding members attached to the substrate. Caged (unactivated) members have a relatively low affinity for receptors of substances that specifically bind to uncaged binding members when compared with the corresponding affinities of activated binding members. Thus, the binding members are protected from reaction until a suitable source of energy is applied to the regions of the surface desired to be activated. Upon application of a suitable energy source, the caging groups labilize, thereby presenting the activated binding member. A typical energy source will be light.

Once the binding members on the surface are activated they may be attached to a receptor. The receptor chosen may be a monoclonal antibody, a nucleic acid sequence, a drug receptor, etc. The receptor will usually, though not always, be prepared so as to permit attaching it, directly or indirectly, to a binding member. For example, a specific binding substance having a strong binding affinity for the binding

member and a strong affinity for the receptor or a conjugate of the receptor may be used to act as a bridge between binding members and receptors if desired. The method uses a receptor prepared such that the receptor retains its activity toward a particular ligand.

Preferably, the caged binding member attached to the solid substrate will be a photoactivatable biotin complex, i.e., a biotin molecule that has been chemically modified with photoactivatable protecting groups so that it has a significantly reduced binding affinity for avidin or avidin analogs than does natural biotin. In a preferred embodiment, the protecting groups localized in a predefined region of the surface will be removed upon application of a suitable source of radiation to give binding members, that are biotin or a functionally analogous compound having substantially the same binding affinity for avidin or avidin analogs as does biotin.

In another preferred embodiment, avidin or an avidin analog is incubated with activated binding members on the surface until the avidin binds strongly to the binding members. The avidin so immobilized on predefined regions of the surface can then be incubated with a desired receptor or conjugate of a desired receptor. The receptor will preferably be biotinylated, e.g., a biotinylated antibody, when avidin is immobilized on the predefined regions of the surface. Alternatively, a preferred embodiment will present an avidin/biotinylated receptor complex, which has been previously prepared, to activated binding members on the surface.

IX. Conclusion

The present inventions provide greatly improved methods and apparatus for synthesis of polymers on substrates. It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reviewing the above description. By way of example, the invention has been described primarily with reference to the use of photoremovable protective groups, but it will be readily recognized by those of skill in the art that sources of radiation other than light could also be used. For example, in some embodiments it may be desirable to use protective groups which are sensitive to electron beam irradiation, x-ray irradiation, in combination with electron beam lithograph, or x-ray lithography techniques. Alternatively, the group could be removed by exposure to an electric current. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

What is claimed is:

1. A polynucleotide analysis apparatus comprising:

a capillary substrate having a surface that is made of a material that is different from the remainder of the capillary, said surface comprising a plurality of different polynucleotides, each of said different polynucleotides occupying a different area of said surface, each of said areas being 10^{-1} cm^2 or less in detectable area, said surface being within the capillary and comprising more than 10 of such polynucleotides, at least some of said polynucleotides bearing fluorescently labeled target molecules excitable with a laser;

an argon laser light source capable of generating light at a wavelength of about 488 nm or less at said surface to excite said fluorescently labeled target molecules, which generate light at a frequency greater than 488 nm;

said laser oriented to direct the laser light at said surface at an angle capable of contacting said target molecules;

a CCD detector oriented in a manner capable of detecting light fluoresced from said target molecules in said areas; and

a data collection, recording and management system for storing fluoresced light intensity, said data collection, recording and management system coupled to said CCD detector, said data collection, recording and management system storing light intensity and converting said light intensity into nucleic acid sequence information.

2. A polynucleotide analysis apparatus in accordance with claim 1 wherein the substrate is translated relative to the laser light.

3. A polynucleotide analysis apparatus in accordance with claim 1 wherein the capillary is composed of quartz.

4. A polynucleotide analysis apparatus in accordance with claim 1 wherein the surface is a gel.

5. A polynucleotide analysis apparatus in accordance with claim 4 wherein the apparatus is used in DNA gel scanning.

6. A polynucleotide analysis apparatus in accordance with claim 4 wherein there are a plurality of capillaries.

7. A polynucleotide analysis apparatus comprising an argon laser light source capable of generating a laser beam providing light at a wavelength of about 488 nm or less to excite fluorescently labeled target molecules attached to said polynucleotides in a substrate to emit excited fluorescent light at a wavelength greater than 488 nm detected by a CCD detector oriented in a manner capable of detecting said emitted fluorescent light from said target molecules in said substrate irradiated by said laser beam, said substrate having more than 10 polynucleotides and each detectable polynucleotide being present in 10^{-1} cm^2 or less in detectable area, said CCD detector coupled to a data collection system for analyzing fluoresced light intensity and converting said light intensity into nucleic acid sequence information.

8. A polynucleotide analysis apparatus in accordance with claim 7 wherein the substrate is a plurality of capillaries.

9. A polynucleotide analysis apparatus in accordance with claim 8 wherein the substrate further comprises a gel or polymer.

10. A polynucleotide analysis apparatus in accordance with claim 8 wherein said apparatus is used for DNA or protein gel scanning.

11. A polynucleotide analysis apparatus in accordance with claim 8 further comprising a translation system including a translation table arranged to support said substrate.

12. A polynucleotide analysis apparatus in accordance with claim 11 including a lens system arranged to focus said laser beam onto said substrate.

13. A polynucleotide analysis apparatus in accordance with claim 8 wherein said fluorescently labeled target molecules are within the capillaries.

14. An apparatus for analyzing nucleic acid binding, comprising:

a substrate that comprises at least 1000 different spheres, beads, or particles having different species of nucleic acids attached thereto, the area of the substrate containing the at least 1000 spheres, beads, or particles being less than 1 cm2, at least some of the nucleic acids being bound to fluorescently labeled target nucleic acids;

a laser energy source to illuminate the fluorescent labels;

a detector to detect a fluorescent label bound to said target nucleic acids; and

a data collection system for storing fluoresced light intensity.

15. An apparatus in accordance with claim 14 wherein the substrate comprises wells, trenches or etched regions.

16. An apparatus in accordance with claim 14 wherein the detector comprises a microscope.

17. An apparatus in accordance with claim 14 wherein the detector comprises a CCD camera.

18. An apparatus in accordance with claim 14 wherein the wavelength of the laser is 488 nanometers or less.

19. An apparatus in accordance with claim 14 wherein the comprises beads.

20. An apparatus in accordance with claim 14 wherein the substrate comprises spheres.

21. An apparatus in accordance with claim 14 wherein the substrate comprises particles.

22. An apparatus in accordance with claim 18 wherein the substrate or its surface may be composed of a polymer, plastic, a resin, silica or silica-based materials, carbon, metals, or inorganic glasses.

23. An apparatus in accordance with claim 18 wherein the substrate or its surface may be composed of a polymer.

24. An apparatus in accordance with claim 18 wherein the substrate or its surface may be composed of silica.

25. An apparatus in accordance with claim 14 wherein the apparatus comprises a translator to move the substrate relative to the detector.

26. An apparatus in accordance with claim 14 wherein there are 10,000 different spheres, beads, or particles.

27. An apparatus for detecting binding of nucleic acids; comprising:

(a) a substrate that comprises at least 1000 different spheres, beads, or particles having different species of nucleic acids attached thereto, the area of the substrate containing the at least 1000 spheres, beads, or particles being less than 1 cm2, at least some of the nucleic acids being bound to fluorescently labeled target nucleic acids

(b) a laser excitation light source;

(c) a detector capable of receiving a signal from the fluorescent labels, the detector comprising a microscope;

(d) a translator to move the substrate relative to the detector; and

(e) a data collection system adapted to receive input from the detector.

28. An apparatus in accordance with claim 27 wherein the detector acquires data every 1 to 100 microns.

29. An apparatus in accordance with claim 27 wherein the detector acquires data every 0.8 to 10 microns.

30. An apparatus in accordance with claim 27 wherein the substrate comprises beads.

31. An apparatus in accordance with claim 27 wherein the substrate comprises spheres.

32. An apparatus in accordance with claim 27 wherein the substrate comprises particles.

33. An apparatus in accordance with claim 27 wherein the substrate or its surface may be composed of a polymer, plastic, a resin, silica or silica-based materials, carbon, metals, or inorganic glasses.

34. An apparatus in accordance with claim 27 wherein there are at least 10,000 different spheres, beads, or particles.

35. A method for screening large numbers of biological polymers, comprising:

providing target nucleic acids;

providing a substrate having an array of at least 1000 different beads, the different beads occupying an area on a substrate of less than 1 cm2, at least some of the different beads having different nucleic acids covalently attached thereto;

contacting the target nucleic acids and the beads so that after contact at least some of the nucleic acids on the beads hybridize to the target nucleic acids further comprising having fluorescently labeled nucleic acids bound thereto;

illuminating the array with a laser energy source to excite the fluorescent labels; and

detecting fluoresced light with a detector that is connected to a data storage system; and

determining which nucleic acids on the beads have bound to target nucleic acids.

36. A method in accordance with claim 35 wherein the detector comprises a microscope.

37. A method in accordance with claim 35 wherein the detector comprises a CCD camera.

38. A method in accordance with claim 35 wherein the substrate or its surface may be composed of a polymer, plastic, a resin, silica or silica-based materials, carbon, metals, or inorganic glasses.

39. A method in accordance with claim 35 wherein the fluorescent label is attached to the target nucleic acid before contact with the beads.

40. A method in accordance with claim 35 wherein the nucleic acids bound to the beads are oligonucleotides.

41. A method in accordance with claim 35 wherein there are 100,000 beads.

42. A method in accordance with claim 35 wherein the detector acquires data every 1 to 100 microns.

43. A method in accordance with claim 35 wherein the detector acquires data every 0.8 to 10 microns.

44. An apparatus in accordance with claim 14 wherein the detector acquires data every 1 to 100 microns.

45. An apparatus in accordance with claim 14 wherein the detector acquires data every 0.8 to 10 microns.

46. A method in accordance with claim 35 comprising providing 100,000 beads.

47. A method in accordance with claim 43 comprising providing 100,000 beads.

48. An apparatus in accordance with claim 27 wherein the area of the substrate is less than 10^{-1} cm^2.

49. A method in accordance with claim 35 wherein the area of the substrate is less than 10^{-1} cm^2.

50. An apparatus in accordance wit claim 27 wherein the area of the substrate is less than 10^{-1} cm^2.

51. A polynucleotide analysis apparatus in accordance with claim 6 wherein there are greater than 100 polynucleotides within the capillaries.

52. An apparatus for detecting binding of nucleic acids; comprising:

(a) a substrate that comprises and at least 1000 different spheres, beads, or particles having different species of nucleic acids attached thereto, the area of the substrate containing the at least 1000 spheres, beads, or particles being less than 1 cm^2, at least some of the nucleic acids being fluorescently labeled;

(b) an excitation light source;

(c) a CCD detector capable of receiving a signal from the fluorescent labels,

(d) a data collection system adapted to receive input from the detector.

53. A method for screening large numbers of biological polymers, comprising:

providing target nucleic acids;

providing a substrate having an array of at least 1000 different beads, the different beads occupying an area

on a substrate of less than 1 cm^2, at least some of the different beads having different nucleic acids covalently attached thereto;

contacting the target nucleic acids and the beads so that after contact at least some of the nucleic acids on the beads hybridize to the target nucleic acids further comprising having fluorescently labeled nucleic acids bound thereto;

illuminating the array with an energy source to excite the fluorescent labels; and

detecting fluoresced light with a CCD detector that is connected to a data storage system; and

determining which nucleic acids on the beads have bound to target nucleic acids.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,646,243 B2
DATED : November 11, 2003
INVENTOR(S) : Pirrung et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

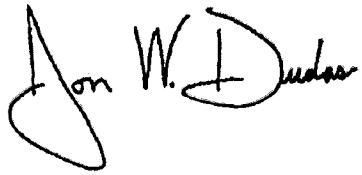
Column 30,
Line 59, "cm2" should read -- cm^2 --;

Column 31,
Line 10, insert -- substrate -- before "comprises";
Lines 34 and 66, "cm2" should read -- cm^2 --;

Column 32,
Line 45, "wit" should read -- with --; and
Line 52, delete "and" after "comprises".

Signed and Sealed this

Twenty-eighth Day of December, 2004



JON W. DUDAS
Director of the United States Patent and Trademark Office